translation no. 5-04

date: | July 1968

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The Utilization of Tissue Cultures for Production of Vaccines Against Venezuelan and American Western Equine Encephalomyeitis Viruses

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Translated from Voprosy Virusologii, v. 2:156-160, 1961, by SPC Elden E. Bwing, Technical Library, Technical Information Division.

Investigations of recent years have shown that the area of spread of the American equine encephalomyelitides is significantly greater than was earlier supposed.

Of particular note are the communications concerning the isolation of the pathegens of the American equina encephalomyelitides in the sestern hemisphere, particularly in Czechoslovakia (6). These data enable us to raise the question about the development of vaccines against the viruses of the American equine escephalomyelitides. The vaccination of laboratory workers is a requirement for work with such contagious agents as the viruses of these diseases.

There is no necessity to emphasize the undoubted advantages of the modern cultural vaccines over the brain and egg (embrye) types.

However, there has not been a single attempt to utilize tissue cultures for the production of vaccines against these viruses, maither in the U.S.S.R. nor in these countries where work with these viruses has been conducted for a long period of time and on a broad scale.

Morks along the line of specific prophylaxis have been limited to the use of brain vaccines for the vaccination of horses (9) and refined embryo vaccines for the vaccination of laboratory workers (8).

As has been shown in many investigations (2, 4, 5, 7), the American equine

ancephalomyelitia viruses propagate in various tissue cultures and build up to high titers in the cultural fluid. Particularly high titers are produced when the viruses propagate in a culture of chick-ambryo fibroblascs. Until now, however, the question about the accumulation of the virus in a cultural fluid without protein has not been investigated, withough this is extremely important to know in the preparation of a vaccine. Another uninvestigated question is whether the issunogenic property of the virus is retained to an adequate degree in the cultural fluid after processing with formalin.

We set for ourselves the task to learn the possibility of utilizing the tissue of lture for the production of vaccines against two viruses: the American western equine encephalomyetitis virus (WEE) and the Venezuelan equine encephalomyetitis virus (VEE).

Meterials and Mathods

The strain of the WEE virus that we used was jut through 40 passages on a culture of chick smbryo fibroblasts; the strain of the WEE virus went through 12 passages on a like culture.

A c-literal field (medium No. 199), which was taken 24 hours after inoculation of the culture, frozen at ~70°6 and kept at ~20°C, was used as the virus-containing material.

A try, minized suspension of chick-embryo fibroblasts was used for the preparation of the tissue culture. The cells were introduced into the matrient medium (1.2 million cells to 1 ml of medium). The resultant suspension was joured into separating flasks (100 ml per flask) or into small bottles (i ml per bottle). A medium of the following composition was utilized: 45% Hanks solution, 45% cow amniotic fluid, 10% ox serum. After a 24-hour insubation at 37°C the cells formed a moselayer. The nutrient medium was drawn off and the sulture was inoculated with the virus; after a one-hour contact the virus was drawn off and the supporting medium No. 199 was added.

A neutralisation test was conducted on both mice and on tissue culture; in the latter instance, 0.1 ms of the virus with the serum was added to a like volume of the ceilular suspension; after one-half hour the mutrient medicus was added. The method for the neutralization on tissue cultures in small bettles has been described in detail previously (1).

Formalin, in an end dilution of 1:4000, was added to the cultural fluid for the inactivation. The incubation was conducted for a period of 5 days at 37°C. A 2.2% solution of sodium bisulface was utilized for the deformalization (1 mi per 20 ml of vectine).

The method for checking the issunogenicity of the vaccine is described below.

Kesuits

In the first series of experiments we set our task to determine how the composition of the medium affects the accumulation of the virus in the cultural fluid and whether it is possible to receive high viral titers with the use of a pretein-free medium. For this purpose three groups of visis (10 visis per group), sontaining a memolayer culture of chick-embryo fibroblasts, were insculated with the WEE virus at 1000 LD per visi. After a one-hour contact the viral suspension was removed and a supporting medium was added to the visie: in the first group - medium No. 199 without serum; in the second - a medium with a hydrolysate of with albumin, containing 2% ox serum; and in the third - a medium containing 70% Hanks solution, 20% ammietic fluid and 10% ox serum. This medium, which was berrowed from Brown's work (3), we shall call Brown's medium. Samples of the cultural fluid were taken directly after the addition of the medium and after each 24 hours. The samples were titrated on mice via intracerébral insculation.

Within 24 hours after inoculation the concentration of the virus in the

This concentration was approximately the same for the vials of all three groups, regardless of the composition of the sequence. Leter, in the vials where sedium No. 199 had been used as the supporting medium the concentration of the virus fell rapidly. Within 40 hours after inoculation it did not extudied and especially the medium with the hydrolysate of milk albumin, high viral titers were being detected in the cultural fluid even later than 40 hours after the inoculation (Table 1).

However, not in a single instance was there a higher viral concentration noted than that which is observed within 24 hours after inoculation, while utilizing medium No. 199. Thus, it was proved possible to use a protein-free cult re medium for the production of vaccine, with the stipulation that the cultural fluto be taken within 24 hours after inoculation.

defore proceeding to the preparation of the vaccine it was necessary to check whether a similar pattern of viral accumulation is observed when working with large volumes of the medium in separating flashs, and whether analogous results are received with the VEE virus. We inoculated two separating flashs (2 for each virus) with the WEE and VEE viruses, with 100,000 LD_{50 for} flash. The viral suspension was drawn off after a one-hour contact and 50 ml of medium to. 199 were saided to each flash. Titration of semples, taken after 24 hours and 48 hours, confirmed the necessity for using the 24-hour cultural fluid for the production of the vaccine, insemuch as the titers of the WEE and VEE viruses in it were 10^{-6} and $10^{-6.77}$, respectively; within 40 hours after insculation the titer of the WEE virus fell to $10^{-4.5}$.

For the inactivation of the virus, we added formalia, in an end dilution

of 1:4000, to the cultural fluid that had been collected from the flasks within 24 hours after the ineculation. The inactivation was conducted at 37°C. In order to study the pattern of the inactivation, we took samples of the cultural fluid directly after the addition of the formalia, and slap after 2 hours, 4 hours and 24 hours. The samples were deformalizized with sodium bisulfate and titrated on mice. The results of these experiments are presented in the diagram. It proved that the WEE and VEE viruses are extremely sensitive to the action of formalin. Within 24 hours after its addition to the cultural fluid the virus could not be detected in the latter. A particularly rapid drop in the titer of the WES and VER viruses occurred during the first two hours of impetivation. We conducted the imactivation during 5 days. Then the vaccines were deformalizated. The completeness of the vaccine's inactivation was checked by means of inoculating mice in the brain. Such batch of vaccine (100 ml) was tested on 75 mice (with the exception of the first batch, when only 20 mice were impoulated). After 4 days ene-third of the mice were stunged and used for a passage; later, still another passage was conducted. There were 4 batches of the VEE vaccine and 3 batches of the WEE vaccine checked.

In one instance, in the insculation with the VRE vaccine, one mouse died; insemuch as we were unsuccessful in isolating the virus from the brain, we evaluate this death as accidental and consider the vaccine as being completely inactivated.

Testing of the immunegenicity of the vaccines was conducted by two
methods: by immunization of rabbits and rate with a subsequent determination
of the entiredy level in the serum, and by means of a direct check of the
protective action of the vaccine on mice.

Two rabbits and 5 rate were impunized with each vectime. The rabbits

were intraperitoneally injected with 5 ml of vaccine three times with an interval of two days. The rate were intraperitoneally injected three times with 2 ml of vaccine with an interval of one day. Within one month after the last immunisation the animals were reismanized s single time, and after two more weeks they were excanguinated. The antibody content in the sers was determined by means of a mentralization test on mice and on tissue culture. In conducting the test on the mice, tenfolo dilutions of the virus were nixed with an equal volume of undiluted serum and, after a one-hour incubation at 37°C, were injected into the brains of the mice in a 0.03-ml volume. The neutralization index was calculated. In the neutralization on tissue culture, four-fold dilutions of the serum were mixed with 100 TGD₅₀ of the virus, and after a one-hour incubation, they were introduced into vials containing 0.1 ml of a suspension of chick-embryo fibroblasts. After 30 minutes, Brown's medium was added.

After a 4%-hour incubation the results were read according to the cytu, athogenic effect. The titer of the serum was Jatermined.

The antibody level in the sera of the immunized animals, particularly in the rabbit sera, proved high. The neutralization indices reached 1000 (table 2).

The titer of the serum from the rabbit immunized with the VEK vaccine proved to be an even 512. There was not a single instance where the neutralization index was below 100, nor where the antibody titer in the serum, which was determined on tissue culture, was below 32.

The immunization of the animals and the subsequent determination of the antibody level in the sera give, however, only an indirect representation of the immunogenicity of vaccines. Therefore we decided to conduct a direct determination of the vaccines' protective strangth. Mive, weighing 7-8 gr., received 0.25 ml spiece of the VEE vaccine. It was administered three times intraperitoneally with an interval of one day. Within 10 days after the last injection, the mice were inoculated intraperitoneally with varying dilutions of the VEE virus.

At the same time the central mice which had not received the vaccine, were inoculated with the same dilutions of the virus. The experiment was twice repeated. Mice suchlings were used in an analogous experiment with the WEE virus. The plan and the results of these experiments are shown in table 3. All of the vaccinated mice remained healthy. As seen in table 3, the VEE vaccine insures high indices of protection (more than 1 million). Unfortunately the low sensitivity of the mice to the intraperitoneal insculation with the WEE virus limits the significance of the resistance test in the work with this virus.

The experimental findings that have been presented indicates the possibility of producing an effective cultural vaccine for the prevention of the contine excephalomyelitides. We do not consider the conditions under which we received our preparation as optimal: we made no attempt to select the best temperature regime during the inactivation period; and we used only one concentration of formulis. All this, however, does not depreciate the fact, but rather emphasizes it, that with the common method of producing a vaccine it is possible to produce on extremely immenogenic preparation that insures the pretection of mice from I million lethal doses in a intraperitoneal insculation. This type of vaccine can be recommended for study as a prophylactic agent against the equine excephalomyelitides.

Conchesions

- 1. The American western and Venezuelan equine encephalosyclitis viruses accumulate in cultural fluid in high titure; with the use of a protein-free medium, however, the viral titer seen drops sharply. It follows, therefore, to use a cultural fluid that has been collected within 24 hours after inoculation for the production of vaccine.
- 2. The cultural formalizated vections against the American western and Venezuelan equins exceptalonyclitic viruses cause the appearance of virus-

neutralizing antibodies in the sera of vaccinated animals and protect mice from the disease when they are given an intraperitoneal injection of up to $1.000,000~{\rm LD_{50}}$ of the virus, thus proving themselves to be highly immangemic preparations.

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TABLE 1 CONCENTRATION OF THE WER VINUS IN THE CULTURAL PLUID MAILE USING DIFFERENT MUTHERF MEDIA

INDONIATING THE INDONIANA COLUMN UNTIL IND. 199	VIEAL BOSE FOR TRE	THE LAPSE PROM	THE VIRAL	THE VIRAL CONCENTRATION IN THE CULTURAL FLUID (LD ₁₀ in 0.03 ml)	LIGAL PUBL
1 106.29 2 103.33 3 101.67 4 100.83		COLLUNE USTIL THE TAKING OF THE SAMPLES (In days)	F. 13	Medius: with the Special of Milk diberties	Mrown's modius
	10 ³ Lb ₅₀	1	106.29	105.83	103.3
		7	103.33	105.33	104.3
		m	101.67	103.67	103.0
•		•	100.63	103.33	Not investigated
9		•	•	102.33	•
		v 9		202.33	

Graph (shown on page 15.)

Inactivation of the WER and WEE viruses with formalin at 37°C. The time, in hours, is plotted along the horizontal and the legarithm of the viral titer, in LD₅₀/ml, is plotted along the vertical.

TABLE 2 THE CONTENT OF VIRUS-HEUTRALIZING ANTIBODY IN THE SERA OF ANTIBALS IDSCRIZED WITH CULTURAL VER AND WEE VACCINES

SERUM	Vinus	SERON TITER IN THE HEUTRALIZATION TEST ON TISSUE CULTURE	REUTRALLATION INDEX, WITH THE TEST CONDUCTED ON MISE
Rabbit	VER	512	102.27
Ret		32	102.11
Rabbit	WEE	12 .	103.14
Rat	••	128	102.44

TABLE 3 TESTING OF THE PROTECTIVE ACTION OF THE VACCINES AGAINST THE VER AND WER VIRUSES

MEN	MICE	A		2110	1101	DILUTION OF THE VIRUS (12)	VIRUS	(35)			India of
		(17 Imoculation) 1.0 2.0 3.0 4.0 5.0 6.0 7.0 4.0	0.1	2.0	0.5	0.4	5.0	0.0	7.0	9.6	PROTECTION
YII.	Vaccinated	0.25 ml		01/0	01/0	0/10 0/10 0/10 0/10 0/10	01/0	01/0			× 23.710
	Control	:				0/10	\$/10	0/10 5/10 7/10 6/10 0/10	01/0	0/10	
722	Vaccinated		1/0	1/0 1/0 1/0 1/0 1/0 1/0 1/0	C/0	1/0	1/0	6/10	1/0		>> 1,000,000
r	Control				12	1/0 1/1 1/1 1/1 1/1	1,1	12	6/1	1/0	
VEE	Vaccinated	, ,	7/0	0/4 0/4 0/4 0/4 0/4	4/0	*/ 0	1/0	1/0			
•	Control		4/4	4/4 4/4 4/4 2/4 0/4 0/4	*	3/2	4,0	1/0			>> 3,160 (1)

Designation: numerator . tile number of deceased pice.

denominator - the number of mice inoculated.